Identification of Bacterial Plant Pathogens Using Multilocus Polymerase Chain Reaction/Electrospray Ionization-Mass Spectrometry

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ABSTRACT

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Polymerase chain reaction/electrospray ionization-mass spectrometry (PCR/ESI-MS, previously known as "TIGER") utilizes PCR with broad-range primers to amplify products from a wide array of organisms within a taxonomic group, followed by analysis of PCR amplicons using mass spectrometry. Computer analysis of precise masses allows for calculations of base compositions for the broad-range PCR products, which can then be compared to a database for identification. PCR/ESI-MS has the benefits of PCR in sensitivity and high-throughput capacity, but also has the distinct advantage of being able to detect and identify organisms with no prior characterization or sequence data. Existing broad range PCR primers, designed with an emphasis on human pathogens, were tested for their ability to amplify DNA of well characterized phytobacterial strains, as well as to populate the existing PCR/ESI-MS bacterial database with base counts. In a blinded panel study, PCR/ESI-MS successfully identified 93% of unknown bacterial DNAs to the genus level and 73% to the species/subspecies level. Additionally, PCR/ESI-MS was capable of detecting and identifying multiple bacteria within the same sample. The sensitivity of PCR/ESI-MS was consistent with other PCR based assays, and the specificity varied depending on the bacterial species. Preliminary tests with real life samples demonstrate a high potential for using PCR/ESI-MS systems for agricultural diagnostic applications.

Plant diseases caused by pathogenic bacteria are particularly devastating because there are no effective postinfection treatments. The long period between introduction of a pathogen and discovery of the resulting disease (from days to years) makes successful eradication or containment of disease very difficult. For example, citrus canker, caused by the bacterium *Xanthomonas citri* subsp. *citri*, was most likely introduced into Florida several years before it was discovered there (18). Moreover, a new disease caused by an emerging, never-before seen pathogen may be initially misdiagnosed, further delaying an appropriate response. Pierce's disease of grape, caused by the fastidious bacterium *X. fastidiosa*, was thought to be caused by a virus for over 20 years (4).

Bacterial plant pathogens are currently identified primarily by phenotypic and immunological methods (5,19). Phenotypic methods, including gram-stain, colony morphology, growth on selective media, and various biochemical reactions, are time consuming and require some experience as well as culturable organisms. Several immunological methods are available for more rapid presumptive identification (5,19), including enzyme-linked immunosorbent assays (ELISA), immuno-fluorescence colony staining (25), and immuno-strip tests (5). All of these assays require characterization of the pathogen to the point that pathogen specific reagents are available. There are currently no known immunoassays that are capable of simultaneously detecting and identifying mixtures of multiple types of bacteria, including unknown organisms. Plant pathogens also have been detected and identified by numerous nucleic acid-based techniques, including Southern blot hybridization and direct nucleotide sequencing. The polymerase chain reaction (PCR) and, more recently real-time PCR, have revolutionized the field of molecular diagnostics. Many PCR based assays have been developed for bacterial plant pathogens (6,12,13,16,18, reviewed in 19). PCR-based diagnostics can be highly specific and are much more sensitive than immunoassays or other nucleic acid-based techniques. However, most PCR assays are designed to detect only a single specific pathogen, and most PCR assays require prior knowledge of at least a portion of the pathogen's genetic sequence, limiting their ability to detect unknown and uncharacterized emerging pathogens. Sequencing based techniques using broad range primers do have the ability to detect multiple pathogens (2), but require significant amounts of labor.

DNA microarray technology represents advancement in molecular diagnostics over PCR-based assays, being able to simultaneously detect numerous pathogens in a single assay. DNA arrays have proven successful in the detection of some pathogens (primarily viruses) important in human health and medicine (11, 23,24), and some plant pathogens (1). However, only a few preliminary studies have been published on the use of DNA array-based technologies for the detection of plant-pathogenic bacteria. Despite the benefits of DNA array-based assays, they, like many

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other nucleic acid-based systems, have the disadvantage of requiring a prior knowledge of at least part of the specific pathogens' genomic sequence. An alternative approach takes advantage of mass spectrometry to determine sequence (8,9). However, like sequencing, these methods are limited in their ability to deal with mixed samples, require specific primers, and are limited in the size of DNA products that can be analyzed. It would be highly beneficial to have diagnostic and surveillance systems that rapidly detect and identify any and all pathogens in a particular sample without prior knowledge of the specific organisms.

The T-5000 biosensor (previously referred to as "TIGER") is designed to rapidly detect and identify emerging pathogens and biothreat agents without prior knowledge of the pathogen's nucleic acid sequence (7). The T-5000 uses broad-range PCR primers that target conserved regions of bacterial genomes, such as ribosomal sequences and conserved elements from essential protein-coding genes (i.e., housekeeping genes). The use of such broad-range priming targets across the widest possible grouping of organisms enables amplification of most species within a group. The strategic breakthrough with the T-5000 biosensor is the use of electrospray ionization-mass spectrometry to analyze the products of broad-range PCR (PCR/ESI-MS). The high mass accuracy and resolution of the PCR/ESI-MS system allows for the precise determination of the molecular mass of the PCR products (10,15). These high precision mass measurements are used to unambiguously derive base compositions (xAxGxCxT) of the PCR products, which then are compared to a database for the identification of the organism. This provides less information than sequencing (exact order of bases is not determined), but allows for a multilocus identification with significantly less time and effort. This paper describes the first application of this technology to plant pathogens, specifically bacterial plant pathogens.

MATERIALS AND METHODS

Bacterial strains and DNA preparation. Ninety-three characterized phytobacterial strains, representing both Gram-negative and Gram-positive species, were analyzed using PCR/ESI-MS (Table 1). Their base compositions were added to the existing PCR/ESI-MS database, which contained over 62,000 bacterial species. No uncharacterized strains were used in the database population, however some plant bacteria were included in the database based only on sequences harvested from GENBANK. All strains were characterized by one or more of the following methods: DNA-DNA similarity, internal transcribed region (ITS) and/or 16S rDNA gene sequence analysis, amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), biochemical characterization, and pathogenicity. Nucleic acid extractions were performed by the Marmur method as described by Schaad et al. (20). DNA concentrations were assessed by spectrophotometry (Perkin Elmer, San Jose, CA) and adjusted to a final concentration of 10 ng/µl.

Broad-range PCR. The outline of PCR/ES-MSI process is briefly demonstrated by Figure 1. All PCR reactions were performed in a 50-µl reaction volume using 96-well microtiter plates. The reaction plates utilized 16 sets of broad-range primers (Table 2) designed for general bacterial surveillance (7). The primer sets are contained in separate wells rather than a multiplex format. Bacterial DNA templates were amplified with each of the 16 primer sets in duplicate plates, with six samples (five samples plus one negative control) to be run per plate. PCR plates were set up using the BioRobot 8000 (Qiagen, Valencia, CA). An ALPS 300 automated plate sealer (ABgene, Epsom, UK) was utilized to seal all PCR plates to avoid contamination and evaporation. PCR was carried out using an Eppendorf Mastercycler ep Thermocycler (Hamburg, Germany). The PCR reaction buffer consisted of 2.5 units of FastStart Taq (Roche, Indianapolis, IN), 1× buffer II, 2.0 mM $MgCl_2$, 0.4 M betaine, 800 µM dNTP mix, and 250 nM propyne containing PCR primers. Prior to PCR each sample was diluted 1:100 with genome dilution buffer (Ibis Biosciences, Carlsbad, CA) to a final concentration of 100 pg/µl. All PCR reaction wells were loaded with 5 µl of DNA, resulting in a concentration of 500 pg of DNA per well. Each PCR plate contained one negative control consisting of genome dilution buffer (Ibis Biosciences). The following PCR cycling conditions were used: 95°C for 10 min followed by 8 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 30 s followed by 37 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 20 s.

Mass spectrometry and base composition analysis. After PCR, an aliquot of each reaction was desalted and purified using an anion-exchange resin protocol (10). The PCR product was transferred to a second 96-well plate containing magnetic beads with an anion-exchange matrix. The negatively charged nucleic acids were retained by an anion-exchange matrix as a series of wash steps were done to remove salts and excess reaction reagents from the well (7). After clean-up, the purified PCR products were eluted from the stationary phase using a methanol-containing buffer (Ibis Biosciences). The purified amplicons were subsequently transferred to a clean 96-well plate to ensure that no beads were withdrawn into the syringe or spray tip.

A Bruker Daltonics microToF (Billerica, MA) mass spectrometer (MS) was used for analyzing the purified DNA. Samples from each reaction well were individually sprayed into the MS using a LEAP autosampler (Carrboro, NC). Internal mass standards and plasmid calibrants were utilized to obtain high mass accuracy of approximately 5 to 10 ppm and provide accurate quantification, respectively. Once the raw spectra were collected, proprietary signal-processing software was used to interpret the mass/charge (m/z) data from the MS and determine the amplicons' molecular mass. Due to the microToF's high mass accuracy (mass measurement error <1 ppm) (15) the amplicon's mass can be very accurately determined and assigned a confident base composition (xA, xT, xC, and xG). Because the bacterial surveillance assay uses 16 primers, there are multiple base counts assigned for each sample from various parts of the genome. When the multiprimer data is combined as a whole, the software can triangulate down to only a few, often one, probable match for pure samples. The base composition of unknown samples is compared to base compositions of other bacteria in a database, allowing for final identification.

Blinded panel. Following the addition of the characterized phytobacteria base composition data to the existing PCR/ESI-MS database, a blinded panel was prepared to evaluate the system's ability to identify these bacteria. Three types of samples were used: single bacterial strains, mixtures of multiple strains, and DNA extracted from infected plant tissue. Citrus seedlings (sweet orange) were inoculated with *X. citri* subsp. *citri* (also known as *X. axonopodis* pv. *citri*). Cabbage plants were inoculated with *X. campestris* pv. *campestris*. Tissue also was taken from an oak tree (Fort Detrick, MD), which was previously confirmed to be infected with *Xylella fastidiosa* by PCR. DNA was extracted from infected tissue using a Qiagen Plant DNeasy extraction kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocols. DNA was diluted and analyzed as previously described. All phytobacteria DNA samples were prepared at the USDA facility, Fort Detrick, MD, and submitted as a blinded panel for analysis at U.S. Army Medical Research Institute for Infectious Disease, Fort Detrick, MD. Following PCR/ESI-MS analysis the PCR/ESI-MS calls were compared to the known ID for system accuracy at the genus, species, and subspecies levels.

Limits of detection and limits of specificity. The limits of detection for PCR/ESI-MS were tested using serial dilutions of *Pseudomonas syringae* pv. *syringae*, *Acidovorax facilis*, *Ralstonia solanacearum*, *Rhodococcus fascians*, and *Xylella fastidiosa*. Serial dilutions of purified DNA were made with a final concentration ranging from 0.05 to 50 ng per reaction. Each sample was

diluted 1:100 with genome dilution buffer and analyzed by PCR/ESI-MS. To confirm reproducibility, samples were run in duplicate. The ability of PCR/ESI-MS to distinguish closely related strains of the same species testing was done using eight strains of *R. solanacearum*, which had been characterized by sequencing of the ITS region and 16S rDNA gene, along with RFLP analysis, biochemical tests, and pathogenicity tests (21).

RESULTS

Ninety-three bacterial strains, representing both Gram-negative and Gram-positive phytobacteria, were selected for addition to the existing PCR/ESI-MS bacterial database. In total, 19 genera of bacteria were included: *Acidovorax* (seven strains), *Agrobacterium* (two), *Arthrobacter* (one), *Burkholderia* (two), *Clavibacter* (four), *Comamonas* (one), *Curtobacterium* (four), *Delftia* (one), *Erwinia* (five), *Herbaspirillum* (one), *Leifsonia* (one), *Pantoea* (one), *Pseudomonas* (four), *Ralstonia* (11), *Rathayibacter* (four), *Rhodococcus* (one), *Xanthomonas* (48), *Xylella* (eight), and *Xylophilus* (two). Several species from each genus, if available, were analyzed, including many important phytopathogenic bacteria.

The broad-based prokaryote-specific primers used for PCR/ESI-MS analysis were designed primarily for human pathogens. Therefore, it was necessary to determine the efficiency of these primers on phytobacteria. Seven of the primer pairs successfully amplified all, or most of the plant bacterial strains tested. These included primer sets for the 16S rDNA (346, 347, 348, and 361), 23S rDNA (349 and 360) and a primer set for the *rpoB* gene (362) (Table 2). Other primer sets (352, 354, 355, 356, 358, 359, 363, 367, and 449) did not amplify the target or generated inconsistent

TABLE 1. Bacterial strains used in this study

Organism	Strain	Host/origin	Source[a]
Acidovorax avenae sp. *avenae*	FC 180; 3403 PAV	Vasey grass; USA	1
Acidovorax avenae sp. *avenae*	FC-320; ATCC19860	Corn; Florida, USA	1
Acidovorax avenae sp. *avenae*	FC-143; ATCC19882	Rice; Japan	1
Acidovorax avenae sp. *cattleyae*	FC-502; Supp364	Phalaenopsis; Japan	1
Acidovorax avenae sp. *citrulli*	FC-247; ATCC29625	Watermelon; USA	1
Acidovorax facilis	FC 208; ATCC 11228	lawn soil; USA	1
Acidovorax konjaci	FC 321; ATCC 33996	Konjac; Japan	1
Comamonas testosterone	FH-55; ATCC11996	Soil; USA	1
Delftia acidovorans deposited as *Comamonas acidovorans*	FC-560; ATCC15668	Soil; Netherlands	1
Agrobacterium vitis	S4	Grape; Hungary	2
Agrobacterium tumefaciens	UBA PF2	Cherry tree crown gall	2
Arthrobacter ilicis	ATCC14264; PDDCC 2607	American holly; USA	3
Burkholderia caryophylli	PC113; ATCC25418	Carnation; USA	1
Burkholderia gladioli pv. *gladioli*	FC-368; PM107; ATCC10248	Gladiolus; USA	1
Clavibacter michiganensis sp. *insidiosus*	FH-37; LMG3660	Alfalfa; USA	1
Clavibacter michiganensis sp. *nebraskensis*	ATCC 27822	Corn; Nebraska, USA	3
Clavibacter michiganensis sp. *sepedonicus*	ATCC33113	Potato; Canada	3
Clavibacter michiganensis sp. *tessellarius*	Vidaver 78203	Wheat/unknown	3
Curtobacterium flaccumfaciens pv. *flaccumfaciens*	LMG3645	Kidney bean; Hungary	3
Curtobacterium flaccumfaciens pv. *violaceum*	ATCC23827	Bean seed; NE; USA	3
Curtobacterium flaccumfaciens pv. *auranticum*	ATCC12813	Bean seed; NE; USA	3
Curtobacterium flaccumfaciens pv. *betae*	FH-3; ICMP 2594	Beet; UK	1
Erwinia amylovora	FE-21	unknown; USA	1
Erwinia amylovora	FE-23	Pear; USA	1
Erwinia carotovora sp. *atroseptica*	NCPPB549	Potato; UK	3
Pantoea agglomerans (synonym) *Erwinia herbicola*	ATCC33243	Cereals; Canada	4
Erwinia persicinus	ATCC35998	Tomato; Japan	4
Erwinia rhapontici	ATCC29283	Rhubarb; England	4
Herbaspirillum rubrisubalbicans	FC-589; ATCC 19308	Sugar cane; USA	1
Leifsonia xyli sp. *cynodontis*	TB1A-2	Bermuda grass	3
Pseudomonas syringae pv. *phaseolicola*	FC-1; C-199	Bean; USA	1
Pseudomonas syringae pv. *syringae*	FC-579; B728	Snap bean; USA	1
Pseudomonas syringae pv. *syringae*	FC-580; 301D	Pear; USA	1
Pseudomonas syringae sp. *oryzicola*	FC-96; PO101	Rice; Hungary	1
Ralstonia solanacearum race 1 bv. 1	FC-326; JR659	Tomato; USA	1
Ralstonia solanacearum race 1 bv. 1	FC-328; JS768	Potato; France	1
Ralstonia solanacearum race 1 bv. 1	FC-329; JS775	Musa; Honduras	1
Ralstonia solanacearum race 1 bv. 1	FC-7; UW275	Melampodium; Costa Rica	1
Ralstonia solanacearum race 1 bv. 3	FC-272; Pe121	Sweet Pepper; Thailand	1
Ralstonia solanacearum race 1 bv. 3	FC-333; SUPP203	Strelitzia; Shizuoka; Japan	1
Ralstonia solanacearum race 1 bv. 3	FC-325; JT526	Tomato; France	1
Ralstonia solanacearum race 1 bv. 3	Se-664	Sesame seeds; Thailand	5
Ralstonia solanacearum race 1 bv. 4	FC-9; UW378	Olive; China	1
Ralstonia solanacearum race 3 bv. 2	FC-540; UW73	Potato; Ceylon	1
Ralstonia solanacearum race unk. bv. N2	FC-330; JS934	Potato; Japan	1
Rathayibacter iranicus	FH-6; LMG3677	Wheat; Iran	1
Rathayibacter rathayi	FH-108; CFPB 2406	Orchard grass; New Zealand	1
Rathayibacter toxicus	FH-99; CS 14; NCPPB 3552	Ryegrass; Australia	1
Rathayibacter tritici	FH-5; NCPPB1857; CT102; ATCC 11403; ICMP2626	Wheat; Egypt	1
Rhodococcus fascians	ATCC12974	Sweet pea; USA	3

(*Continued on next page*)

[a] Sources: 1, N. W. Schaad, International Collection of Phytopathogenic Bacteria (ICPB) maintained by ARS-USDA, FDWSRU, Fort Detrick, MD; 2, T. J. Burr; Cornell University, Geneva, NY; 3, A. Vidaver, Department of Plant Pathology, University of Nebraska, Lincoln, NE; 4, G. H. Lacy Laboratory for Molecular Biology of Plant Stress Department of Plant Pathology, Physiology, and Weed Science Virginia Polytechnic Institute and State University Blacksburg, VA; and 5, Niphone Thaveechai Department of Plant Pathology Faculty of Agriculture Kasetsart University, Bangkok, Thailand.

results, which were most likely due to a nonspecific amplification. Initial analysis of the database suggested that the working primer sets would be sufficient for identification of plant bacteria to at least the genus level, so a blinded panel was prepared to test the diagnostic capability of PCR/ESI-MS for phytobacteria.

Blinded panel. Sixty-four bacterial DNAs were prepared for a blinded panel. Fifty-six samples contained individual bacterium, eight samples contained mixtures of multiple bacterial strains. In addition, DNA from a healthy oak tree and a no-template sample were included as negative controls. The data from the blinded panel was processed and identifications assigned to each sample. Fifty-two of the single isolates were identified correctly to the genus level (93%). Forty-one of the single isolates were correctly identified to the species and/or subspecies level (73%). Four of the single isolates were incorrectly identified (Table 3).

Eight samples in the blinded panel contained multiple bacterial species. Seven of these mixtures contained three strains, and one contained five strains. The identity of the mixture samples and the number of organisms per sample were unknown to the researcher performing the analysis. In all of the mixtures PCR/ESI-MS was able to correctly identify most of the strains to the genus level (22 of 26 in total), and in many cases PCR/ESI-MS was able to correctly identify strains at the species/subspecies level (mixture H, Table 4, Fig. 2). In mixtures B, C, F, G, and H, all strains were identified to at least the genus level. In mixtures A and E two out of three strains were recognized by the system. In other mixtures

TABLE 1. (*Continued from preceeding page*)

Organism	Strain	Host/origin	Source[a]
Xanthomna aracearum pv. *syngonii*	X1674	Syngonium; unknown	4
Xanthomnas citri sp. *citri*	FB-1342	Citrus; Indonesia	1
Xanthomonas euvesicatoria	FB-1290; ATCC 11633	Pepper; USA	1
Xanthomonas albilineans	FB-1306; ATCC33915	Sugarcane; Fiji	1
Xanthomonas alfalfae pv. *citrumelonis*	FB-1275; D. Gabriel #3048	Citrus sp.; USA	1
Xanthomonas alfalfae pv. *citrumelonis*	FB-1274; D. Gabriel #4600	Citrus sp.; USA	1
Xanthomonas aracearum pv. *anthurii*	LMG695	Anthurium; Brazil	4
Xanthomonas arboricola pv. *juglandis*	ATCC 49083	English walnut; New Zealand	4
Xanthomonas arboricola pv. *pruni*	ATCC 19316; VPI-93; FB-1303	Japanese plum; New Zealand	4
Xanthomonas axonopodis pv. *axonopodis*	FB-1083; ATCC19312	Carpetgrass; Colombia	1
Xanthomonas axonopodis pv. *begoniae*	FB-1313; ATCC49082	Begonia; New Zealand	1
Xanthomonas axonopodis pv. *dieffenbachiae*	FB-1320; ATCC23379	Dieffenbachia; USA	1
Xanthomonas axonopodis pv. *glycines*	ATCC 11766	Soybean; India	4
Xanthomonas axonopodis pv. *manihotis*	VPI-20; ATCC 49073	Cassava; Brazil	4
Xanthomonas axonopodis pv. *phaseoli*	VPI-13; ATCC 49119	Bean; USA	4
Xanthomonas axonopodis pv. *vasculorum*	FB-1297; ATCC 35938	Sugarcane; Mauritus	1
Xanthomonas axonopodis pv. *vasculorum*	FB-1322; ATCC 13901	Sugarcane; Puerto Rico	1
Xanthomonas axonopodis pv. *vignicola*	VPI-19; ATCC 11648; FB-1305; XV18	Cowpea; USA	4
Xanthomonas axonopodis pv. *vitians*	FB-1309; ATCC19320	Lettuce; USA	1
Xanthomonas axonopodis sp. *alfalfae*	XA129; Med. 2	Alfalfa; Sudan	1
Xanthomonas campestris	FB-1000	Cabbage; USA	1
Xanthomonas campestris pv. *incanae*	FB-1310; ATCC 13462	Tenweeks stock; USA	1
Xanthomonas campestris pv. *uppalii*	FB-639; ATCC 11641	Morning glory; India	1
Xanthomonas carpentii pv. *papavericola*	FB-5; ATCC 14179	Shirley poppy; USA	1
Xanthomonas citiri sp. *malvacearum*	FB-1235; "H"	Cotton; USA	1
Xanthomonas codiaei	FB-1242; LMG8678	Garden croton; USA	1
Xanthomonas cucurbitae	FB-1054	Watermelon; USA	1
Xanthomonas fragariae	FB-1243; ATCC 33239; VPI-117	Strawberry; USA	1
Xanthomonas fuscans	XP37; ATCC13464	Pea bean; USA	1
Xanthomonas fuscans sp. *aurantifolii*	FB-1259; LMG9179	Citrus limon; Argentina	1
Xanthomonas fuscans sp. *aurantifolii*	FB-1261; LMG9182	Key lime; Mexico	1
Xanthomonas hederae	FB-1298; ATCC 9653	English ivy; USA	1
Xanthomonas hortorum pv. *pelargonii*	FB-1325; ATCC 8721	Pelargonium; unknown	1
Xanthomonas hyacinthi	FB-1245; ATCC 19314	Garden hyacinth; The Netherlands	1
Xanthomonas oryzae pv. *oryzae*	VPI-33; ATCC 35933	Rice; India	4
Xanthomonas oryzae pv. *oryzicola*	FB-1312	Rice; unknown	4
Xanthomonas perforans	Xv938	Tomato; USA	4
Xanthomonas pisi	FB-1247; ATCC 35936	Garden pea; Japan	1
Xanthomonas sp. pv. *convolvuli*	FB-635; LMG685	Bindweed; India	1
Xanthomonas sp. pv. *eucalypti*	VPI-38; ATCC 49081; XE104	Lemon Eucalyptus: Australia	4
Xanthomonas translucens pv. *arrhenatheri*	VPI-28; ATCC 33803	Tall oatgrass; Switzerland	4
Xanthomonas translucens pv. *graminis*	VPI-27; ATCC 29091	Orchard grass; Switzerland	4
Xanthomonas translucens pv. *phlei*	VPI-29; ATCC 33805	Timothy; Norway	4
Xanthomonas translucens pv. *poae*	VPI-30; ATCC 33804	Rough bluegrass; Switzerland	4
Xanthomonas translucens pv. *secalis*	VPI-98; ATCC 49078; XT129	Spring rye; Canada	4
Xanthomonas translucens pv. *translucens*	VPI-32; ATCC 19319	Barley; USA	4
Xanthomonas translucens pv. *undulosa*	VPI-31; ATCC 35935	Rivet wheat; Canada	4
Xanthomonas vesicatoria	FB-1022; ATCC 35937	Tomato; New Zealand	1
Xylella fastidiosa	FK-79; #89	Mulberry; unknown	1
Xylella fastidiosa	FK-61; Ann1; ATCC 700598	Oleander; USA	1
Xylella fastidiosa sp. *fastidiosa*	FK-44; Traver	Grape; USA	1
Xylella fastidiosa sp. *fastidiosa*	FK-57; MAPLE	Maple; USA	1
Xylella fastidiosa sp. *multiplex*	FK-46; AC8	Almond; USA	1
Xylella fastidiosa sp. *pauca*	FK-32; CVC09-2N	Citrus; Brazil	1
Xylella fastidiosa sp. *pauca*	FK-67; CVC 08-01	Citrus; Brazil	1
Xylella fastidiosa sp. *sandii*	FK-53; OLF#1	Oleander; USA	1
Xylophilus ampelinus	FB-1178	Grape; S. Africa	1
Xylophilus ampelinus	FJ-3; 60002	Grape; S. Africa	1

one bacterial strain seemed to dominate (i.e., mixture D). The most difficult mixture was a combination of five closely related *X. fastidiosa* strains (mixture G), which was correctly identified as multiple *X. fastidiosa* strains. PCR/ESI-MS was able to correctly identify the sources of four out of the five strains as typical of those isolated from citrus, grape, almond, and oleander, but was unable to distinguish the fifth strain from maple (data not shown).

Infected plant samples. In addition to DNA extracted from pure bacterial cultures, DNA was extracted from several plants that were artificially and naturally infected with phytobacteria for PCR/ESI-MS analysis. PCR/ESI-MS was able to detect and correctly identify infections of *X. fastidiosa* in oak and *X. citri* from infected citrus (Table 5). Mock-inoculated and healthy citrus plants were recognized as negative by PCR/ESI-MS. Internal calibrants were successfully amplified by the reaction, confirming that PCR conditions were adequate. The host plant DNA was amplified in healthy plant samples, indicating that the broad range primers will amplify plant mitochondrial or chloroplast genomes. However, the base composition analysis was able to clearly distinguish the bacterial pathogens from the healthy background signal, allowing for identification of the organism.

Limits of detection and limits of specificity. Five different strains were tested in four serial dilutions to test the limits of sensitivity for the PCR/ESI-MS system. Three strains (*P. syringae*, *R. solanacearum*, and *R. fascians*) were detected at a concentration of 5 pg/reaction. Strains of *A. facilis* and *X. fastidiosa* had a lower detection limit of 0.5 pg/reaction. The amount of DNA per cell varies with respect to the growth conditions (14), and has been determined for several bacterial species (14,22). An average DNA content per cell in pure bacterial culture is about 12 fg, which indicates that PCR/ESI-MS consistently detects samples containing the equivalent of 400 cells (5 pg DNA/well) and in some cases 42 cells (0.5 pg/well) (Table 6).

Using the broad range primer sets described previously, PCR/ESI-MS was also able to distinguish multiple strain variants of *R. solanacearum* used to determine the system's specificity. Table 7 shows the base counts resulting from analysis of nine *R. solanacearum* strains. PCR/ESI-MS was able to differentiate all but two of the nine strains used in this study. This demonstrates the system's ability to parse closely related organisms based on the multiprimer data produced by this assay. However, the PCR/ESI-MS primer panel used in this study was less able to distinguish subspecies of genus *Xanthomonas* and pathovars of *P. syringae* (Table 3).

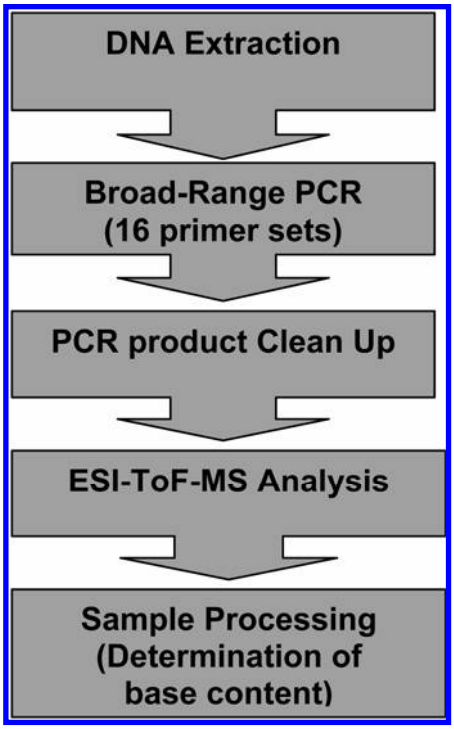

Fig. 1. Flow chart of polymerase chain reaction/ectrospray ionization-mass spectrometry (PCR/ESI-MS).

DISCUSSION

The necessity of early and accurate detection of plant pathogens cannot be overemphasized. There is clearly a need for diagnostic assays that are capable of detecting a broad range of pathogens simultaneously and accurately, with the capability of high-throughput processing. In addition, it would be useful that such an assay be able to detect and identify unknown organisms, even if no preexisting sequence data or diagnostic primers are available. PCR/ESI-MS represents a novel approach that has been successful in addressing these concerns for a number of human pathogens (3,17). For example, Sampath et al. (17) used PCR/ESI-MS to analyze several isolates of coronaviruses, many of which did not have a genome sequence record in GenBank. Nevertheless, they were able to amplify all test viruses and experimentally determine their base compositions. This highlights the usefulness of this method even with samples for which genome sequence is not known.

Our study represents the first detailed use of the PCR/ESI-MS system in the agricultural arena. It is important to note that the primer panel used in this study was developed with human pathogenic bacteria in mind, and some of the initial broad-range primer sets would not necessarily be expected to amplify and/or distinguish phytobacteria. PCR/ESI-MS testing determined that nine of the sixteen broad-range primers were either ineffective or inconclusive for amplification of phytobacteria. However, using the other seven primer sets, which consistently produced amplicons from all phytobacteria tested, PCR/ESI-MS correctly identified 93% of phytobacterial samples in a blinded panel to the genus level, and 73% at the species or subspecies level (Table 3). The limits of detection for PCR/ESI-MS was within the range of conventional PCR based assays (Table 5). This represents a significant advance in broad-range detection of phytobacteria.

There are two key elements to the power of PCR/ESI-MS. First, is the ability to amplify and identify a bacterial organism in a sample. Second, PCR/ESI-MS has the ability to detect and

TABLE 2. Broad-range primers for bacteria used for polymerase chain reaction/electrospray ionization-mass spectrometry analysis (7)

Primer pair numbers[a]	Genes	Projected bacterial targets
346, 347, 348, 361	16S rDNA	All
349, 360	23S rDNA	All
354	*rpoC*	Bacterioidetes, Fusobacteria, Spirochaetes, Bacilli, Proteobacteria α/β/γ
363	*rpoC*	Proteobacteria α/β
358	*valS*	Some representatives of
359	*rpoB*	γ-Proteobacteria: *Erwinia*, *Pantoaea*, *Pectobacterium*
362	*rpoB*	Proteobacteria α/β
367	*tufB*	Some representatives of β-Proteobacteria: *Eikenella*, *Neisseria*, *Achromobacter*, *Bordella*, *Burkholderia*, *Ralstonia*
356, 449	*rplB*	Clostridia, Fusobacteria, Bacilli, and ε-Proteobacteria (*Campylobacter*, *Helicobacter*, *Wolinella*)
352	*infB*	*Bacilli*
355	*sspE*	*Bacillus cereus*

[a] Primer pairs that were consistently effective for amplifying and distinguishing phytobacteria are shown in bold.

identify multiple bacteria in the same sample. In the blinded panel, PCR/ESI-MS demonstrated the ability to diagnose multiple species in mixtures of phytobacteria. When the bacterial samples in the mixture were not closely related, PCR/ESI-MS could cleanly distinguish all isolates (e.g., mixtures B, C, and H, Table 4). The ability of PCR/ESI-MS to distinguish multiple bacteria in a single sample is based on the system's ability to identify the component bacteria individually (as in mixtures D and E), as well as the relatedness of the component mixtures. Mixture G was a combination of five closely related *Xylella fastidiosa* isolates. PCR/ESI-MS made a call of multiple *X. fastidiosa* strains, but a closer examination of the results demonstrated that PCR/ESI-MS had successfully distinguished four of the five subspecies in the sample (data not shown).

Perhaps most intriguing are the results from infected plant tissues. Infected plants represent more complex systems, including not only the high likelihood of endophytic bacteria, but also the possibility of amplified products generated from mitochondrial and chloroplast genomes. Despite the added complexity of these samples PCR/ESI-MS was able to correctly identify bacterial pathogens in infected citrus, cabbage, and oak tissues.

A key facet of the PCR/ESI-MS system is triangulation, the use of multiple broad-range primer sets to generate base count data

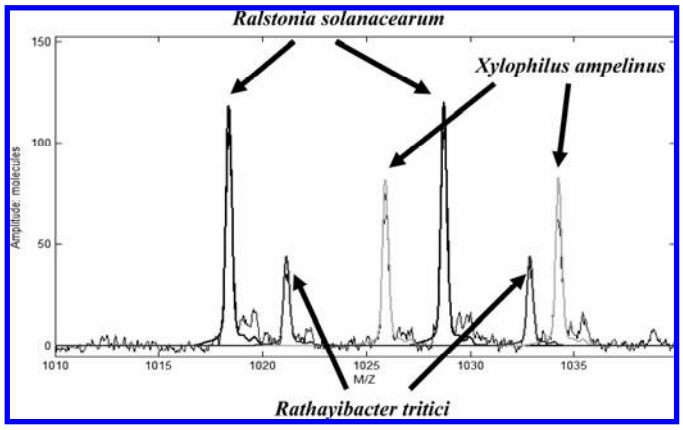

Fig. 2. Polymerase chain reaction/ectrospray ionization-mass spectrometry (PCR/ESI-MS) analysis of three bacteria strains in a single sample. Purified DNAs from three species were combined and PCR/ESI-MS analyzed as a single sample, the MS trace from a single primer set is shown below. The relative quantity of product is shown on the Y axis, mass of individual products is shown on the X axis. The precise mass is used to determine base content for the PCR products. Each bacterial strain generates two peaks, one for each complementary strand of amplified DNA.

TABLE 3. Results of polymerase chain reaction/electrospray ionization-mass spectrometry (PCR/ESI-MS)-blinded panel analysis of individual strain samples

	Organism	PCR/ESI-MS call[a]
Correct to species level	*Acidovorax avenae* (3 strains)	*Acidovorax avenae*
	Acidovorax facilis	*Acidovorax facilis*
	Acidovorax konjaci	*Acidovorax konjaci*
	Agrobacterium tumefaciens	*Agrobacterium tumefaciens*
	Agrobacterium vitis	*Agrobacterium vitis*
	Arthrobacter ilicis	*Arthrobacter ilicis*
	Burkholderia caryophylli	*Burkholderia caryophylli*
	Clavibacter michiganensis sp. *nebraskensis*	*Clavibacter michiganensis*
	Comamonas testosterone	*Comamonas testosteroni*
	Delftia (Comamonas) acidovorans	*Comamonas acidovorans*
	Erwinia amylovora (2 strains)	*Erwinia amylovora*
	Erwinia carotovora subsp. *atroseptica*	*Erwinia carotovora* supsp. *atroseptica*)
	Erwinia chrysanthemi	*Erwinia chrysanthemi*
	Erwinia rhapontici	*Erwinia rhapontici*
	Leifsonia xyli sp. *Cynodontis*	*Leifsonia xyli* sp. *cynodontis*
	Pseudomonas syringae pv. *syringae* (2 strains)	*Pseudomonas syringae* pv. *syringae*
	Pseudomonas syringae pv. *phaseolicola*	*Pseudomonas syringae* pv. *tomato*
	Ralstonia solanacearum (6 strains)	*Ralstonia solanacearum*
	Rathayibacter rathayi	*Rathayibacter rathayi*
	Rathayibacter tritici	*Rathayibacter tritici*
	Rhodococcus fascians	*Rhodococcus fascians*
	Xanthomonas axonopodis (2 strains)	*Xanthomonas axonopodis*
	Xanthomonas campestris	*Xanthomonas campestris*
	Xylella fastidiosa (6 strains)	*Xylella fastidiosa*
	Xylophilus ampelinus (2 strains)	*Xylophilus ampelinus*
Correct to genus level	*Erwinia carotovora* subsp. *carotovora*	Multiple calls: *Erwinia herbicola*, *Pectobacterium carotovorum* (synonym: *Erwinia carotovora*), *Serratia plymuthica*
	Erwinia persicinus	*Erwinia rhapontici*
	Herbaspirillum rubrisubalbicans	Multiple calls: *H. frisingense*, *H. rubrisubalbicans*, *H. seropedicae*
	Pantoaea agglomerans	Multiple *Buttiauxella* sp., *Pantoea agglomerans*, *Citrobacter freundii*
	Xanthomonas citri	*Xanthomonas axonopodis*
	Xanthomonas aracearum pv. *anthurii*	*Xanthomonas axonopodis*
	Xanthomonas hyacinthi	*Xanthomonas axonopodis*
	Xanthomonas sp. pv. *convolvuli*	*Xanthomonas axonopodis*
	X. aracearum pv. *syngonii*	*Xanthomonas axonopodis*
	Xanthomonas translucens pv. *arrhenatheri*	*Xanthomonas axonopodis*
	Xanthomonas translucens pv. *graminis*	*Xanthomonas axonopodis*
Incorrect	*Rathayibacter iranicus*	No call
	Rathayibacter toxicus	No call
	Erwinia carotovora subsp. *carotovora*	*Serratia marcescens*
	Curtobacterium flaccumfaciens pv. *betae*	*Agrobacterium tumefaciens*
	Healthy plant DNAs	No call
	Negative control	Blank

[a] "No call" designates a sample in which PCR/ESI-MS did not identify a bacterial species but PCR products were amplified from some primer sets; "blank" designates samples in which no product was amplified.

TABLE 4. Multiple pathogen results[a]

Mixture of strains	Species tested[b]	PCR/ESI-MS call[b]	Effectiveness at genus level
Mixture A	*Acidovorax avenae*	*Acidovorax facilis*	2/3
	Comamonas testosteroni	*Comamonas testosteroni*	
	Agrobacterium vitis	None[c]	
Mixture B	*Acidovorax konjaci*	*Acidovorax konjaci*	3/3
	Comamonas acidovorans	*Comamonas acidovorans*	
	Agrobacterium tumefaciens	*Agrobacterium tumefaciens*	
Mixture C	*Arthrobacter ilicis*	*Arthrobacter ilicis*	3/3
	Burkholderia andropogonis	*Burkholderia thailandensis*	
	Clavibacter michiganensis sp. *nebraskensis*	*Clavibacter michiganensis*	
Mixture D	*Erwinia amylovora*	*Chromobacterium violaceum*	1/3
	Herbaspirillum rubrisubalbicans	*Herbaspirillum seropedicae*	
	Leifsonia xyli sp. *cynodontis*	*Clavibacter michiganensis*	
Mixture E	*Ralstonia solanacearum* (two strains)	*Ralstonia solanacearum* (two strains)	2/3
	Pseudomonas syringae pv. *syringae*	None[c]	
Mixture F	*P. syringae* pv. *phaseolicola*	*Pseudomonas alcaligenes*	3/3
	Pseudomonas syringae pv. *syringae*	*Pseudomonas alcaligenes*	
	Erwinia amylovora	*Erwinia amylovora*	
Mixture G	*Xylella fastidiosa* from almond	Multiple *Xylella fastidiosa*	5/5
	Xylella fastidiosa from grape		
	Xylella fastidiosa from oleander		
	Xylella fastidiosa from maple		
	Xylella fastidiosa from citrus		
Mixture H	*Ralstonia solanacearum*	*Ralstonia solanacearum*	3/3
	Rathayibacter iranicus	*Rathayibacter iranicus*	
	Xylophilus ampelinus	*Xylophilus ampelinus*	

[a] Purified DNAs from multiple bacteria were mixed at equal ratios and analyzed.
[b] Species tested indicates the source strains and PCR/ESI-MS call shows the bacteria identified by polymerase chain reaction/electrospray ionization-mass spectrometry (PCR/ESI-MS).
[c] None indicates that this bacterial strain was not identified in the mixture.

TABLE 5. Results from infected plant tissue[a]

Organism	PCR/ESI-MS call[b]	Real-time PCR (+/–) (positive/negative)[c]
Citrus inoculated with *Xanthomonas axonopodis* pv. *aurantifolii* ("B")*	*Xanthomonas axonopodis*	+
Citrus inoculated with *Xanthomonas axonopodis* pv. *citri* (A^w)	No call	–
Citrus inoculated with *Xanthomonas citri*	*Xanthomonas citri*	+
Citrus inoculated with *Xanthomonas citri*	*Xanthomonas citri*	+
Citrus inoculated with *Xanthomonas alfalfae* pv. *citrumelosis*	No call	–
Citrus inoculated with *Xanthomonas alfalfae* pv. *citrumelosis*	No call	–
Healthy citrus (sweet orange)	No call	–
Cabbage inoculated with *Xanthomonas campestris* pv. *campestris*	*Xanthomonas campestris*	Not tested
Cabbage inoculated with *Xanthomonas campestris* pv. *campestris*	*Xanthomonas campestris*	Not tested
Oak tree infected with *Xylella fastidiosa*	*Xylella fastidiosa*	+

[a] DNA was extracted from greenhouse-inoculated plants, except for the *Xylella fastidiosa*-infected oak sample, which was taken from a previously tested tree.
[b] No call designates a sample in which polymerase chain reaction/electrospray ionization-mass spectrometry (PCR/ESI-MS) was unable to identify a bacterial species but PCR products were amplified from some primer sets.
[c] In most cases, the presence of infecting bacteria was checked using pathogen-specific real-time PCR assays.

TABLE 6. Limits of detection for polymerase chain reaction/electrospray ionization-mass spectrometry

	DNA amount (pg) per well/ corresponding mean cell counts[a]			
Organism	50/4166	5/417	0.5/42	0.05/4
Pseudomonas syringae	+	+	–	–
Acidovorax facilis	+	+	+	–
Ralstonia solanacearum	+	+	–	–
Rhodococcus fascians	+	+	–	–
Xylella fastidiosa	+	+	+	–

[a] A successful amplification positive identification is indicated by a plus sign (+). No amplification or not enough product to make a successful identification is indicated by a minus sign (–).

from multiple loci. The broad-range primer set was designed with human pathogens as the primary target, and not all of the primers would necessarily be expected to consistently prime phytobacteria. For example, four of the primer sets amplify bacilli and related bacteria; as such they are not useful for phytobacteria (Table 2). Seven of the primer sets worked consistently for phytobacteria. Despite this limitation, the assay was very successful at identifying phytobacteria to the genus level, but in some cases lacked the level of specificity to make species or subspecies calls correctly. PCR/ESI-MS was able to clearly distinguish several strains of *R. solanacearum* when nine primer sets were taken into consideration. This suggests that the development of a few phytobacterial broad-range primer sets should enhance the abilities and uses of PCR/ESI-MS for phytobacterial identification.

A closer look at individual samples suggests that the existing broad-range primer sets may be biased toward *Enterobacteriaceae*, a family that includes human pathogens from genera *Escherichia*, *Salmonella*, *Yersinia*, and *Serratia*, as well as plant pathogens: *Erwinia*, *Pantoea*, and *Pectobacterium*. In blinded panel analysis PCR/ESI-MS sometimes identified phytobacteria as related *Enterobacteriaceae*, for example the *Erwinia carotovora* sp. *carotovora* that was incorrectly identified as *Serratia marcescens*. This is probably due to a combination of factors, including the nature of the broad-range primers as well as the emphasis on human pathogens in the PCR/ESI-MS database.

TABLE 7. Limits of specificity for polymerase chain reaction/electrospray ionization-mass spectrometry (PCR/ESI-MS)[a]

Strain (r/bv)	Primer pair number (gene targets)								
	347 16S	346 16S	348 16S	361 16S	360 23S	349 23S	363 *rpoC*	362 *rpoB*	358 *valS*
JR659 (1/1)	[27 32 26 29]	[30 29 25 15]	[26 34 29 30]	[28 31 25 19]	[32 37 27 26]	[23 27 22 20]	[20 28 37 17]	[19 35 20 18]	No prime
JS768 (1/1)	[27 32 26 29]	[30 29 25 15]	[26 34 29 30]	[28 31 25 19]	[32 37 27 26]	[23 27 22 20]	[20 28 37 17]	[19 35 20 18]	No prime
JS775 (1/1)	[27 32 26 29]	[30 29 25 15]	[34 31 29 25]	[28 31 25 19]	[10 39 46 27]	[23 27 22 20]	[20 28 37 17]	[20 34 20 18]	No prime
JS934 (?/N2)	[27 32 26 29]	[30 29 26 14]	[26 34 29 30]	[28 31 25 19]	[32 37 27 26]	[23 27 22 20]	[16 36 29 21]	[19 33 21 19]	No prime
PE121 (1/3)	[27 32 26 29]	[30 29 25 15]	[26 34 29 30]	[28 31 25 19]	[32 37 27 26]	[23 27 22 20]	[16 36 31 19]	[19 35 20 18]	No prime
Se664 (1/3)	[27 32 26 29]	[30 29 25 15]	[26 34 29 30]	[28 31 25 19]	[32 37 27 26]	[23 27 22 20]	[16 36 31 19]	[19 35 20 18]	[23 40 30 23]
UW73 (3/2)	[27 32 26 29]	[30 29 25 15]	[26 34 29 30]	[28 31 25 19]	[19 27 33 43]	[23 27 22 20]	[20 28 37 17]	[20 34 20 18]	[23 40 30 23]
UW275 (1/1)	[27 32 26 29]	[30 29 25 15]	[34 31 29 25]	[28 31 25 19]	[33 36 26 27]	[24 26 21 21]	No data	[18 36 21 17]	[23 40 30 23]
UW378 (1/4)	[27 32 26 29]	[30 29 25 15]	[26 34 29 30]	[28 31 25 19]	[32 37 27 26]	[23 27 22 20]	[16 36 31 19]	[19 35 20 18]	[23 40 30 23]

[a] Nine strains of *Ralstonia solanacearum* were analyzed by PCR/ESI-MS. The strain identifier along with race and biovar (r/bv) designations are shown in the first column. The nine primer sets used along with target genes are shown at the top. The base composition for individual fragments is shown as xAxGxCxT in the boxes corresponding to strain and primer set. Diagnostic base counts are shaded. This demonstrates the triangulation facet of PCR/ESI-MS, where no single primer pair is sufficient to distinguish closely related bacterial strains, but the combined data from several primer sets can successfully distinguish related strains.

Differences between the present taxonomic name and the nomenclature of the PCR/ESI-MS databases entries cause issues. For example, *Erwinia carotovora* subsp. *atroseptica* was called simply *Erwinia carotovora*, but closer examination determined that the strain designation had been truncated in the PCR/ESI-MS database, and the system had actually correctly identified the sample (strain ID was one of *Erwinia carotovora* subsp. *atroseptica*).

Clearly, as with all databases, the quality and size of the database will directly affect the success of the assay. As more phytobacterial samples are analyzed the PCR/ESI-MS database will improve for phytobacteria identification. In addition, development of additional broad range primers that focus on plant bacteria will improve the system for use in agriculture. An additional benefit of this system is the ability to reprocess older data as the database is updated. Essentially, this system provides multiple ways to revisit data and make identifications, whether error existed as a result of the database, processing, or uncovering a variable strain that has not been seen before.

Our results indicate that PCR/ESI-MS has the capability to consistently detect and identify phytobacteria to at least the genus level. The ability to identify any unknown phytobacteria to the genus level is a significant breakthrough. In addition, PCR/ESI-MS is capable of identifying roughly 70% of tested bacteria to the species level. This level of identification would have previously required multiple PCR tests, ELISA assays, or significant characterization. PCR/ESI-MS can analyze three 96-well plates (18 samples) within a typical 8-h work day, giving multilocus diagnostic information that would take much longer using conventional sequencing. The results provide genus/species ID without needing additional analysis (e.g., sequence assembly and/or BLAST). The initial investment for the PCR/ESI-MS instrument is significant, but once in place the cost of diagnosis is no more than the cost of PCR reagents plus some additional proprietary items (Ibis Biosciences Inc.). As with most new technologies, the cost of the instrumentation is coming down as the technology improves. The database is available with purchase of the instrument from the manufacturer. Furthermore, a high rate of successful identification at the species/subspecies and/or strain level suggests that PCR/ESI-MS has the capacity to meet any requirements for specificity. Augmenting existing primer sets with primer sets aimed at phytobacteria should result in even more accurate identification by PCR/ESI-MS. PCR/ESI-MS has multiple pathogen detection capacity for the cost of several PCR reactions. PCR/ESI-MS also has the theoretical capacity to assist in preliminary classification of unknown bacteria as well, as base counts for unknowns can be used to determine closest relatives in the database, much as unknown sequences can be compared to known sequences in existing sequence databases. PCR/ESI-MS has clear applications in the fields of plant pathogen detection, ecology, and taxonomy.

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LITERATURE CITED

1. Bystricka, D., Lenz, O., Mraz, I., Dedic, P., and Sip, M. 2003. DNA microarray: Parallel detection of potato viruses. Acta Virol. 47:41-44.
2. Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Bandela, A. M., Cardenas, E., Garrity, G. M., and Tiedje, J. M. 2007. The ribosomal database project (RDP-II): Introducing *myRDP* space and quality controlled public data. Nucleic Acids Res. 35(Database issue):D169-D172.
3. Ecker, D. J., Sampath, R., Blyn, L. B., Eshoo, M. W., Samant, V., Russell, K., Freed, N., Barrozo, C. Wu, J., Rudnick, K., Desai, A., Moradi, E., Hannis, J. C., Harrell, P. M., Massire, C. Hall, T. A., Jiang, Y., Ranken, R., Drader, J. J., White, N., McNeil, J. A., Crooke, S. T., Hofstadler, S. A. 2005. Rapid identification and strain-typing of respiratory pathogens for epidemic surveillance. Proc. Natl. Acad. Sci. USA 102:8012-8017.
4. Freitag, J. H., and Frazier, N. W. 1954. Natural infectivity of leafhopper vectors of Pierce's disease virus of grape in California. Phytopathology 44:7-11.
5. Hampton, R. O., Ball, E. M., De Boer, S. H., eds. 1990. Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. American Phytopathological Society, St. Paul, MN.
6. Henson, J. M., and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. Annu. Rev. Phytopathol. 31:81-109.
7. Hofstadler, S. A., Sampath, R., Blyn, L. B., Eshoo, M. W., Hall, T. A., Jiang, Y., Drader, J. J., Hannis, J. C., Sannes-Lowery, K. A., Cummins, L. L., Libby, B., Walcott, D. J., Schink, A., Massire, C., Ranken, R., Gutierrez, J., Manalili, S., Ivy, C., Melton, R., Levene, H., Barrett-Wilt, G., Li, F., Zapp, V., White, N., Samant, V., McNeil, J. A., Knize, D., Robbins, D., Rudnick, K., Desai, A., Moradi, E., and Ecker, D. J. 2005. TIGER: The Universal Biosensor. Int. J. Mass Spectrom. 242:23-41.
8. Honisch, C., Chen, Y., Mortimer, C., Arnold, C., Schmidt, O., van den Boom, D., Cantor, C., Shah, H., and Gharbia, S. 2007. Automated comparative sequence analysis by base-specific cleavage and mass spectrometry for nucleic acid-based microbial typing. Proc. Natl. Acad. Sci. USA 104:10649-10654.
9. Jackson, G. W., McNichols, R. J., Fox, G. E., and Wilson, R. C. 2006. Universal bacterial identification by mass spectrometry of 16S ribosomal cleavage products. Int. J. Mass Spectrom. 261:218-226.
10. Jiang, Y., and Hofstadler, S. A. 2003. A highly efficient and automated method of purifying and desalting PCR products for analysis by electrospray ionization mass spectrometry. Anal. Biochem. 316:50-57.
11. Lapa, S., Mikheev, M., Shchelkunov, S., Mikhailovich, V., Sobolev, A., Blinov, V., Babkin, I., Guskov, A., Sokunova, E., Zasedatelev, A.,

Sandakhchiev, L., and Mirzabekov, A. 2002. Species-level identification of orthopoxviruses with an oligonucleotide microchip. Clin. Microbiol. 40:753-757.
12. Liu, W. J., Chang, Y. S., Wang, C. H., Kou, G. H., and Lo, C. F. 2005. Microarray and RT-PCR screening for white spot syndrome virus immediate-early genes in cycloheximide-treated shrimp. Virology 334:327-341.
13. Louws, F. J., and Cuppels, D. 2001. Molecular Techniques. Pages 321-333 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd ed. N. W. Schaad, J. B. Jones, and W. Chun, eds. American Phytopathological Society, St. Paul, MN.
14. McCoy, W. F., and Olson, B. H. 1985. Fluorometric determination of the DNA concentration in municipal drinking water. Appl. Environ. Microbiol. 49:811-817.
15. Muddiman, D. C., Anderson, G. A., Hofstadler, S. A., and Smith, R. D. 1997. Length and base composition of PCR-amplified nucleic acids using mass measurements from electrospray ionization mass spectrometry. Anal. Chem. 69:1543-1549.
16. Perreten, V., Vorlet-Fawer, L., Slickers, P., Ehricht, R., Kuhnert, P., and Frey, J. 2005. Microarray-based detection of 90 antibiotic resistance genes of Gram-positive bacteria. J. Clin. Microbiol. 43:2291-2302.
17. Sampath, R., Hofstadler, S. A., Blyn, L. B., Eshoo, M. W., Hannis, J. C., Harrell, P. M., Massire, C., Hall, T. A., Levene, H. M., Samant, V., Neuman, B., Buchmeier, M. J., Jiang, Y., Ranken, R., Drader, J. J., Griffey, R. H., McNeil, J. A., Crooke, S. T., and Ecker, D. J. 2005. Rapid identification of emerging infectious diseases: A coronavirus. Emerg. Infect. Dis. 11:373-379.
18. Schaad, N. W., Frederick, R. D., Shaw, J., Schneider, W. L., Hickson, R., Petrillo, M. D., and Luster, D. G. 2003. Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. Annu. Rev. Phytopathol. 41:305-324.
19. Schaad, N. W., Jones, J. B., and Chun, W. (eds.) 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd ed. American Phytopathological Society, St. Paul, MN.
20. Schaad, N. W., Postnikova, E., Lacy, G., Fatmi, M., and Chang, C. J. 2004. *Xylella fastidiosa* subspecies: *X. fastidiosa* subsp. *piercei*, subsp. nov., *X. fastidiosa* subsp. *multiplex* subsp. nov., and *X. fastidiosa* subsp. *pauca* subsp. nov. Syst. Appl. Microbiol. 27:290-300. (Erratum 27:763).
21. Thammakijjawat, P., Thaveechai, N., Kositratana, W., Chunwongse, C., Frederick, R. D., and Schaad, N. W. 2001. Genetic analysis of *Ralstonia solanacearum* strains from different hosts in Thailand using PCR-restriction fragment length polymorphism. Kasetsart J. Nat. Sci. 35:397-408.
22. Tranvik, L. J. 1997. Rapid fluorometric assay of bacterial density in lake water and seawater. Limnol. Oceanogr. 42:1629-1634.
23. Wang, D., Coscoy, L., Zylberberg, M., Avila, P. C, Boushey, H. A., Ganem, D., and DeRisi, J. L. 2002. Microarray-based detection and genotyping of viral pathogens. Proc. Natl. Acad. Sci. USA 99:15687-15692.
24. Wang, D., Urisman, A., Liu, Y.-T., Springer, M., Ksiazek, T. G., Erdman, D. D., Mardis, E. R., Hickenbotham, M., Magrini, V., Eldred, J., Latreille, J. P., Wilson, R. K., Ganem, D., and DeRisi, J. L. 2003. Viral discovery and sequence recovery using DNA microarrays. PLoS Biol. 1:257-260.
25. Van Vuurde, J. W. L. 1990. Immunofluorescence colony staining. Pages 295-305 in: Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. R. Hampton, E. Ball, and S. De Boer, eds. American Phytopathological Society, St. Paul, MN.